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# Histone acetylation modulates uncoupling protein1 expression in brown adipocytes

Anubama Rajan

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# HISTONE ACETYLATION MODULATES UNCOUPLING PROTEIN 1 EXPRESSION IN BROWN ADIPOCYTES

by

ANUBAMA RAJAN

Under the Direction of Bingzhong Xue

## ABSTRACT

Uncoupling protein 1 (UCP1) is a classical feature of brown adipocytes and understanding its regulatory mechanism will help in the development of a pharmacological approach for obesity and associated metabolic diseases. The epigenetic regulation of UCP1 in brown adipocytes is not completely understood. Our study is focused on histone deacetylases (HDACs), which are set of enzymes that bring about changes in gene expression pattern by changing the histone acetylation status. Our data suggest that inhibition of Class-I HDACs can increase the expression of UCP1 in brown and white adipocytes; whereas inhibition of Class-II HDACs can decrease the UCP1 expression in brown adipocytes. Thus, by pharmacologically targeting specific HDAC enzymes, it might be possible to modulate UCP1 expression and thermogenic function in brown and white adipocytes. This will help burning excessive energy in the form of heat and in turn promote reduction of body weight, alleviate obesity and associated metabolic diseases.

INDEX WORDS: Uncoupling protein 1, Brown fat, Epigenetics, Histone deacetylase (HDAC), HDAC inhibitors, Obesity

HISTONE ACETYLATION MODULATES UNCOUPLING PROTEIN 1 EXPRESSION IN  
BROWN ADIPOCYTES

by

ANUBAMA RAJAN

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Masters of Science

in the College of Arts and Sciences

Georgia State University

2014

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2014

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BROWN ADIPOCYTES

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## DEDICATION

அகர முதல எழுத்தெல்லாம் ஆதி

பகவன் முதற்றே உலகு.

*Just as the alphabet 'A' is the beginning of all letters,  
so also, God is the beginning for this universe.- Thiruvalluvar.*

I first thank almighty, the God for giving me the strength, wisdom and everything to complete my work.

I sincerely dedicate my work to my loving parents, Rajan Rangaswamy and Mythili Rajan, who have always supported me and never left my side. To my brother, Anandan Rajan who has always been my strength and I sincerely appreciate his efforts in helping me in his own special way. I would like to extend a special gratitude to my brother, Balaraman Rajan for constantly encouraging and inspiring me during my research.

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## 1 INTRODUCTION

Obesity is a medical condition which is characterized by excessive storage and accumulation of body fat. This accumulation of adiposity causes several additional metabolic disorders such as: diabetes, hypertension, dyslipidemia, fatty liver (non-alcoholic) syndrome and cardio-vascular diseases. The co-morbidities associated with obesity pose a severe threat to human life and quality of living. Thus, a treatment to obesity will not only promote weight loss but also improve the quality of human life.

Adipose tissue has a fundamental role in both distribution and storage of energy and thus contributes equally to energy homeostasis and metabolism. Adipose tissue is broadly classified into two main types: white adipose tissue (WAT), which preserves the energy in the form of tri-glycerides and brown adipose tissue (BAT), which wastes energy in the form of heat using uncoupling protein 1 (UCP1). This process is called non-shivering thermogenesis and is driven by uncoupling oxidative phosphorylation in mitochondria<sup>1</sup>. UCP1 can also be induced in white fat. Such cells that express UCP1 in WAT are called beige cells<sup>2</sup>. As energy is dissipated by this special protein-UCP1 in the form of heat, it is of special interest and target for scientists to study its mechanisms to treat obesity and promote weight loss. UCP1 expression can be up-regulated using stimulatory responses such as prolonged cold exposure and stimulation by adrenergic receptors<sup>3</sup>. Recent studies have shed insight into the molecular mechanisms behind UCP1 induction in WAT. One such study have stated how positive regulators like peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1 $\alpha$ ) and Peroxisome proliferator-activated receptor gamma co-activator 1-beta (PGC1 $\beta$ ), PR domain containing 16 (PRDM16), and negative regulators like

Retinoblastoma (Rb), receptor interacting protein 140 (RIP140) contributes towards transcriptional regulation of UCP1<sup>12</sup>.

PGC1 $\alpha$  and PGC1 $\beta$  are two transcriptional co-activators which are closely related to each other and they play a vital role in up-regulation of fatty acid oxidation genes. They are also known to increase mitochondrial biogenesis<sup>18</sup>. PRDM16 is a classical gene in brown fat fate determination, which increases the expression of PGC1 $\alpha$ , and interacts with important regulators of brown fat function, including PGC1 $\alpha$ , PGC1 $\beta$ , PPAR $\alpha$  and PPAR $\gamma$ , thereby promoting brown adipocyte phenotype and thermogenic function<sup>24</sup>. Higher levels of PGC1 $\alpha$  and PGC1 $\beta$  mRNA expression are necessary for expression of UCP1 and are inversely correlated with obesity<sup>18</sup>. Recent studies also state that the interaction between PPAR $\gamma$  and PGC1 $\alpha$  greatly augments the activity of PPAR $\gamma$  in up-regulation of UCP1 expression. This evidence throws light on the importance of PGC1 $\alpha$  and PGC1 $\beta$  in regulation of brown fat phenotype, as well as UCP1 expression.

On the other hand, it is fairly necessary to understand the repressive mechanisms of UCP1. The retinoblastoma protein (Rb) belongs to retinoblastoma gene family, is one of the classical repressors of UCP1 expression. Rb has an ability to switch between the WAT and BAT fate and is involved in cell cycle and differentiation<sup>42</sup>. Rb protein phosphorylation or any other functional inactivation of Rb protein has been correlated with induction of brown fat fate and higher expression of UCP1 expression in WAT<sup>16</sup>. These studies demonstrate a repressive role of Rb1 in UCP1 expression. Interactions between Rb and FOXC2 are postulated to be involved in a signaling cascade that increases the level of a UCP1 regulatory protein, Protein Kinase A-R1 $\alpha$ <sup>17</sup>.

Great effort has been devoted to unravel the regulation of brown fat function by these signaling pathways. The epigenetic regulation and mechanisms of UCP1 expression, however, are largely unexplored. Through this study, we mainly addressed the questions behind the epigenetic control mechanisms of UCP1. Understanding these mechanisms will help in promoting brown fat function and induction of brown fat mechanisms in white adipocytes to promote weight loss.

Epigenetics is the study of heritable modifications in the patterns of gene expression caused by mechanisms other than alterations in the actual DNA sequence itself<sup>4</sup>. This is either due to DNA methylation or histone modifications including acetylation, ubiquitination, methylation, ADP-ribosylation, phosphorylation and sumoylation. Epigenetic control of obesity has been previously reported in the literature. Recent research has demonstrated that histone 3 lysine 9 [H3-K9] specific demethylase Jhdm2a, plays an important role in obesity by controlling the expression of PPAR $\alpha$  and UCP1<sup>15</sup>. In addition, the pattern of CpG island methylation of the enhancer regions of UCP1 is found to be specific to the tissues and thus alters the expression pattern of the UCP1 protein in different tissues<sup>6</sup>. Furthermore, nuclear hormone co-repressor, RIP 140 is involved in increasing the assembly of histone methyltransferase on UCP1 enhancer regions. This specifically methylates the CpG islands of the enhancer regions of UCP1 and its co-activators, leading to UCP1 suppression<sup>7</sup>. All of these validate the significance of epigenetics in the control of adipose tissue metabolism and function. The role of HDAC modulation of UCP1 gene expression in brown adipocytes, however, remains unidentified.

HDACs are a set of enzymes, which acts on histones to remove acetyl groups from them. This modification, enhances the ability of histones to wrap around the DNA more tightly. Inhibiting HDAC causes up-regulation whereas activating HDAC causes down-regulation of genes. HDACs are divided into four different super families: Class I to IV. Class I is comprised of HDAC- 1, 2, 3 and 8, whereas Class II-a includes HDAC-4, 5, 7, and 9, and Class II-b includes HDAC 6 and 10. Class IV includes HDAC 11. HDAC III includes the sirtuins, which uses  $\text{NAD}^+$  as cofactor and hence different from the rest of HDACs<sup>8,9</sup>. In the current project, we mainly focused on Class I and II HDACs.

Our preliminary data indicate that TSA (pan HDAC inhibitor) down-regulates UCP1 expression. In contrary, a recent study has revealed that injection of Class I HDAC inhibitors in mice increases mitochondrial activity, ameliorates obesity and insulin resistance in skeletal muscles and increases UCP1 expression in brown adipose tissue<sup>10</sup>. On one hand, this provides confirmation that chromatin modification using pharmacological agents could play an important role in treatment of obesity. On the other hand, these seemingly contradictory effects of the pan-HDAC inhibitor TSA and the class I HDAC inhibitor on oxidative pathway and UCP1 expression may be due to the action of different classes of HDACs in the body. The precise mechanism by which different classes of HDACs regulate UCP1 expression is not clear. Thus, we studied the contrasting roles of this epigenetic mechanism in UCP1 expression in order to provide more insight towards formulation of new therapeutics for obesity and diabetes.

Therefore, in this project, we analyzed the roles of different classes of HDACs in the regulation of brown adipocyte UCP1 expression and function. We hypothesize that there are two

different regulatory mechanisms in UCP1 expression by HDACs. Inhibiting the Class I HDACs potentially up-regulated UCP1 expression whereas inhibiting the Class II HDACs down-regulated the UCP1 expression. We also studied the mechanisms underlying the differential regulation of brown adipocyte UCP1 expression and function by the different HDACs.



## **2. AIM AND HYPOTHESIS**

The overall aim of this study is to identify the mechanisms of epigenetic regulation in UCP1 expression by histone acetylation.

### **2.1 Specific aims**

1. To evaluate the effect of Class-I HDAC inhibition on UCP1 regulation and brown fat function
2. To study the role of Class-II HDAC inhibition on UCP1 expression and brown fat function

### **2.2 Hypothesis**

We hypothesize that there are two different regulatory mechanisms of UCP1 expression by HDACs. Inhibition of the Class I HDACs can potentially up-regulate UCP1 expression and on the other hand inhibition of the Class II HDACs can down-regulate the UCP1 expression.

### 3 MATERIALS AND METHODS

#### 3.1 Chemical reagents

All HDAC inhibitors, MS-275, MC 1568 and TSA (Trichostatin A) was obtained from selleckchem.com. The working concentrations of these HDAC inhibitors are: TSA- 500nM, MS-275- 5 $\mu$ M, MC-1568- 30 $\mu$ M<sup>10</sup>.

#### 3.2 Cell culture

Brown adipocytes cell lines, HIB1B and BAT1 and white adipocyte cell line 3T3-L1 were used. HIB1B cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) along with 10% Fetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (PS) antibiotics at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. 3T3-L1 cells were grown in high glucose DMEM along with 10% New Born Calf Serum and 1% PS antibiotics at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. BAT1 cells were grown in DMEM/F-12 nutrient mixture along with 10% FBS and 1% PS antibiotics at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Undifferentiated HIB1B cells and differentiated HIB1B and BAT1 adipocytes were then pre-treated with HDAC inhibitors for 30 minutes followed by norepinephrine (HIB1B) or isoproterenol (BAT1) stimulation for 4 and 3 hours respectively for induction of UCP1 expression. Differentiated 3T3-L1 cells were treated with MS-275 for various time and then cells were harvested for RNA expression measurement.

### **3.3 Differentiation of pre-adipocyte cell lines**

#### ***3.3.1 3T3L1 adipocyte differentiation***

The 3T3L1 cells were grown until post confluent in DMEM high glucose medium, 10% calf serum and 1% PS. After post confluence 2-3 days, the differentiation was induced using the mixture containing: DMEM medium, 10% FBS, 1% PS, insulin (800nM), isobutyl-1-methylxanthine (500 $\mu$ M) and dexamethasone (1 $\mu$ M) for 2 days. After the differentiation, the cells were maintained in 800nM insulin medium with DMEM, 10% FBS and 1% PS for another 2- 4 days. After complete differentiation of cells, they were grown in regular medium with DMEM, 10% FBS and 1% PS for additional 4- 8 days.

#### ***3.3.2 HIB1B brown adipocyte differentiation***

The HIB1B cells were allowed to grow confluent and the differentiation medium containing DMEM medium, 10% FBS, 1% PS, insulin (800nM), isobutyl-1-methylxanthine (500 $\mu$ M) and dexamethasone (1 $\mu$ M), indomethacin (100 $\mu$ M), thyroid hormone (T3) (1nM) were added to induce differentiation of cells for 3 days. Followed by this, the medium were changed to maintenance medium containing DMEM medium, 10% FBS, 1% PS, insulin (800nM) and T3 (1nM) until 8 days.

#### ***3.3.3 BAT1 differentiation***

The BAT1 cells were allowed to grow until they are 90% confluent. These cells were then be induced for differentiation for 48 hours using the differentiation mix containing: DMEM/F-12 medium, 10% FBS, 1% PS, insulin (20nM), isobutyl-1-methylxanthine (500 $\mu$ M)

and dexamethasone (0.5 $\mu$ M), indomethacin (125 $\mu$ M), T3 (1nM). The cells were then switched to maintenance medium containing DMEM/F-12 medium, 10% FBS, 1% PS, insulin (20nM) and T3 (1nM). The cells differentiated completely in another 5-6 days.

### **3.4 RNA Isolation and quantitative RT-PCR**

Total RNA were isolated and extracted from cells using Tri-Reagent. The quantification of messenger RNAs of the gene of interest were done using real time-PCR using TaqMan One-step RT-PCR master mix (Applied BioSystems, Foster City, CA) and Stratagene Mx300P system (Stratagene, La Jolla, CA). The concentration of the mRNA used were between 8ng to 50ng and the mRNA quantitation were normalized by the corresponding cyclophilin mRNA measurement. TaqMan primer/probes for all genes were bought from Applied Biosystems<sup>15</sup>. The primer sequence for all the genes are provided in Table1.

### **3.5 siRNA Knockdown**

For knockdown of gene expression in HIB1B cells using siRNA, Reverse Transfection procedure were followed. In the first step, RNAi duplex-Lipofectamine™, RNAiMAX complexes were prepared. For this, 6 pmol RNAi duplex was diluted in 100  $\mu$ l Opti-MEM® I Medium containing no serum and gently mixed. 1.5  $\mu$ l of Lipofectamine™ RNAiMAX were added and the obtained mix was incubated for 10-20 minutes at RT. Next, the cells which were allowed to grow to 90% confluence were trypsinized and these cells were diluted in the growth medium without antibiotics. The concentration of the cells were optimized to  $1 \times 10^5$  cells/well. This will ensure that 500  $\mu$ l has the appropriate amount of cells to provide 30-50% confluence after plating. Then the mixture containing RNAi duplex - Lipofectamine™ RNAiMAX

complexes and cells were plated. The cells were incubated for 24-72 hours at 37°C in a CO<sub>2</sub> incubator. They were then be pre-treated with HDAC inhibitors for 30 minutes followed by 4 hours of adrenergic stimulation (nor-epinephrine treatment). Then the gene expression was analyzed.

For siRNA knock down of gene expression in BAT-1 cells, Electroporation technique were used. The cells obtained from Day 4 differentiation were trypsinized and diluted to the concentration of  $3 \times 10^6$  cells/plate in the medium without antibiotics. Based on manufacture's instruction from Lonza (Amaxa Cell Line Nucleofector Kit L) 400µL of Nucleofector solution L was added. After this, 20pmol of siRNA was added per sample. This mixture will then be electroporated using Amaxa biosystems. Immediately after electroporation, the mixture was transferred to a fresh 50 ml tube and mixed with 500µl of maintenance medium/well and plated. The cells were incubated at 37°C in a CO<sub>2</sub> incubator for additional 2 days. The cells were then be pre-treated with HDAC inhibitors for 30 minutes followed by 3 hours of adrenergic stimulation (isoproterenol treatment). Then the gene expression was analyzed.

### **3.6 Chromatin Immuno-Precipitation assay**

At the 6<sup>th</sup> day of differentiation, the cells were first with HDAC inhibitors for 30 minutes and followed by adrenergic stimulation. After treatment, the cells were fixed with 1% of formaldehyde. Protease inhibitors such as: 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1µg/ml pepstatin A are added to ice cold PBS and this was used to wash the fixed cells. The cells were scrapped and collected in a conical tube. After a brief centrifugation, the pellet was suspended in SDS lysis buffer containing 1% SDS, 10mM EDTA and 50mM Tris. The cell

lysate was sonicated to obtain genomic DNA fragment of length 200–1000 bp. These lysate were then centrifuged, and the supernatant of the sample was collected. Followed by this, the supernatant was immuno-precipitated with the antibodies against acetyl histone 3 or antibodies against specific lysine residues on H3. The immuno-complex which underwent immune-precipitation were washed and reverse cross-linked using NaCl. Followed by this the samples are digested with protease K. The DNA was isolated using phenol/chloroform extraction method. The concentration of DNA was measured using Nano drop and subjected to PCR amplification of the promoter regions of UCP1, Rb1, and PGC1 $\alpha$  genes<sup>13</sup>. All the antibodies were obtained from Abcam and Millipore. Sybr Green master mix for qRT-PCR was purchased from Applied BioSystems, Foster City, CA and Stratagene Mx300P system (Stratagene, La Jolla, CA) were used to measure the gene expression pattern. The primers for PCR were obtained from Integrated DNA Technologies and the primer sequence are provided in Table 2.

**Table 1: Primer sequence for TaqMan primer and probes**

Gene	Sequence
UCP1	FP: CACCTTCCCGCTGGACACT RP: CCCTAGGACACCTTTATACCTAATGG Probe: AGCCTGGCCTTCACCTTGGATCTGA
PGC1 $\alpha$	FP: CATTTGATGCACTGACAGATGGA RP: CCGTCAGGCATGGAGGAA Probe: CGTGACCACTGACAACGAGGCC
PGC1 $\beta$	FP: AGGAAGCGGCGGGAAA RP: CTACAATCTCACCGAACACCTCAA Probe: AGAGATTTCTGAATGTATACCACACGGCCTTCA
COX-1	FP: TTTTCAGGCTTCACCCTAGATGA RP: GAAGAATGTTATGTTTACTCCTACGAATATG Probe: CATGAGCAAAAGCCCACTTCGCCA
ACOX-1	FP: AAGAACTCCAGATAATTGGCACCTA or RP: AGTGGTTTCCAAGCCTCGAA Probe: CGGAGATGGGCCACGGAATCAT or ATGAGTTCCGTGGCCCATCTCCG
Cyclophilin	FP: GGTGGAGAGCACCAAGACAGA RP: GCCGGAGTCGACAATGATG Probe: ATCCTTCAGTGGCTTGTCCCGGCT

**Table 2: Primer sequence for ChIP analysis**

Gene	Sequence
UCP1 enhancer region	FP: CTCCTCTACAGCGTCACAGAGG RP: AGTCTGAGGAAAGGGTTGA
UCP1 start region	FP: CCCACTAGCAGCTCTTTGGA RP: CTGTGGAGCAGCTCAAAGGT
PGC1 $\alpha$ Mef2	FP: GCTCGCTGCATTTCTTTCTT RP: CCCCACAGACTCAAAAACCA
PGC1 $\alpha$ CRE	FP: CAAAGCTGGCTTCAGTCACA RP: AAAAGTAGGCTGGGCTGTCA
Rb1 promoter region	FP: TACTTGGGTTCGAGTCCTCTGCCAG RP: AGTTGGCCGTGTTTCATGCG

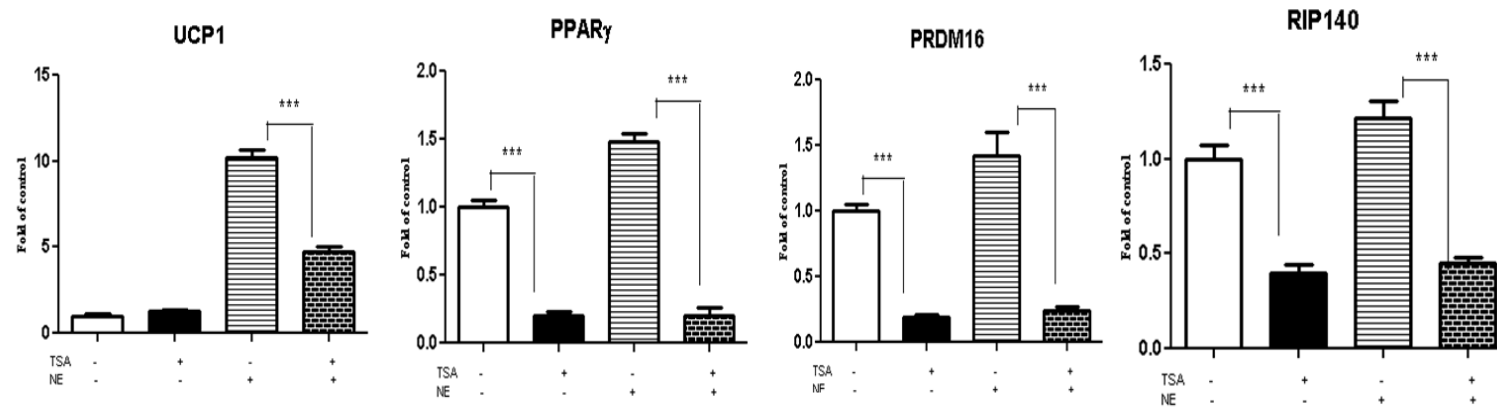


## 4 RESULTS

### 4.1 Role of pan HDAC inhibitors in brown adipose tissue gene regulation

To examine how different HDACs regulate brown fat metabolism and function, we initially studied the effect of a pan HDAC inhibitor, TSA on H1B1B cell lines. The cells were pre-treated with TSA for 30 minutes followed by norepinephrine (adrenergic agonist) stimulation for 4 hours. After the treatment, a significant decrease of UCP1 expression was observed. Genes which are known to regulate of brown fat function such as: PPAR $\gamma$ , PRDM16 also showed a significant decrease. We also saw a decrease in RIP140, which is known as a negative regulator of UCP1 expression<sup>12</sup> (Fig: 1A). In contrast, we also observed a robust increase in PGC1 $\alpha$  and PGC1 $\beta$  which are known as transcriptional co-activators of UCP1<sup>12</sup>. Acetyl Co-A oxidase-1 (ACOX-1), which is involved in  $\beta$ -oxidation of fatty acids also showed a significant increase. Cell death- inducing DFFA- like effector a (CIDEA), which is postulated as a brown fat specific gene playing an important role in lipolysis and thermogenesis also increased after treatment with TSA<sup>36 and 37</sup> (Fig: 1B). SAHA, another pan inhibitor of HDACs showed similar effect to that of TSA and hence the rest of the experiments were continued with TSA as a pan inhibitor (Fig: 2). Similar results were observed for differentiated H1B1B cells when treated with TSA (Data not shown)

1-A



1-B

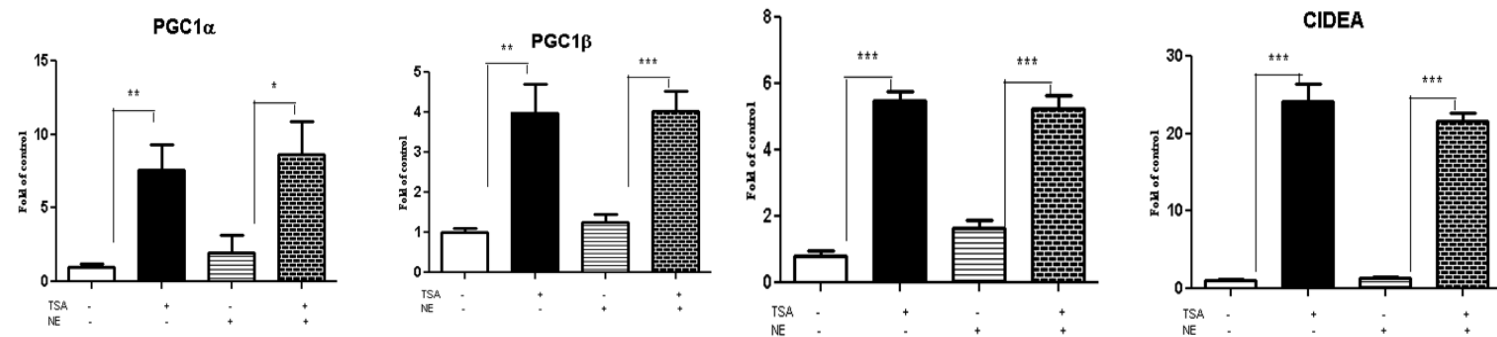


Fig 1: Effect of TSA, a pan HDAC inhibitor on H1B1B cell lines using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (norepinephrine) for 4 hours to observe changes in thermogenic and regulatory genes. 1A) Down-regulated genes 1B) Up-regulated genes. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=4-6). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

**Figure 1: Effect of TSA on H1B1B cells.**

2

## UCP1

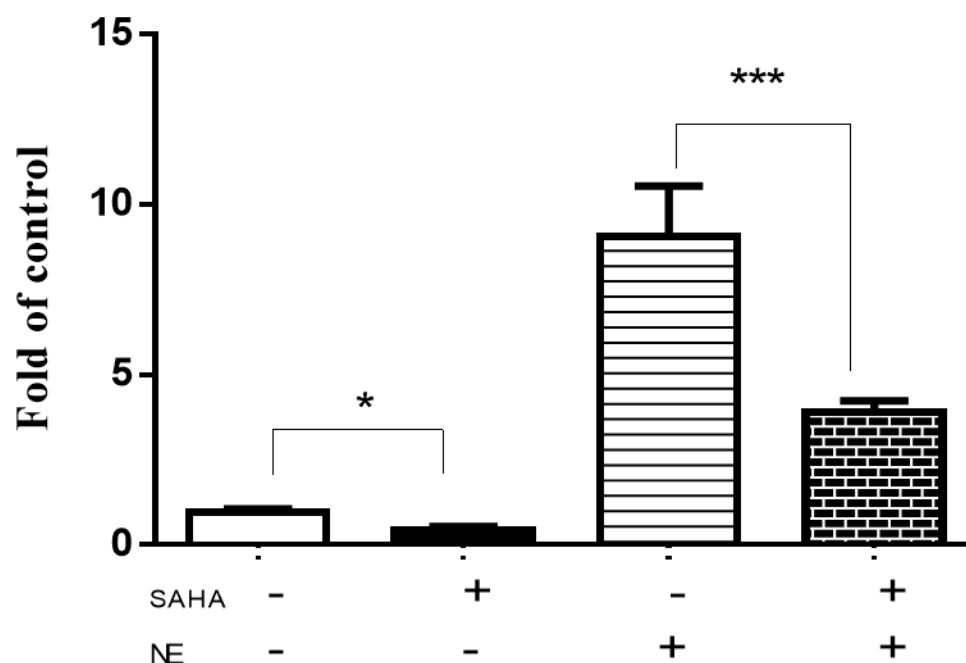


Fig 2: Effect of SAHA, a pan HDAC inhibitor on UCP-1 expression in H1B1B cell lines using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (norepinephrine) for 4 hours to observe changes in UCP1 expression. Values in the experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=4-6). \*P < 0.05, \*\*\*P < 0.001

**Figure 2: Effect of SAHA on H1B1B cells.**

### 4.2 Contrasting effect of Class-I and Class-II HDAC inhibition on UCP1 expression

We next sought to identify how the specific classes of HDACs modulate gene expression profile in brown fat metabolism. For this, we pre-treated H1B1B cells with Class-I specific inhibitor, MS-275 and Class-II specific HDAC inhibitor, MC-1568 followed by norepinephrine stimulation. We observed a strikingly contrast effect on UCP1 expression. While Class-I HDAC inhibition showed a significant increase of UCP1 expression, opposite to that of the pan-HDAC

inhibitors, TSA and SAHA, the Class-II HDAC inhibition showed a significant decrease of UCP1, similar to that of TSA and SAHA (Fig: 3A and 3B). This contrasting effect of different HDACs suggests that different HDACs may regulate UCP1 gene expression in different ways.

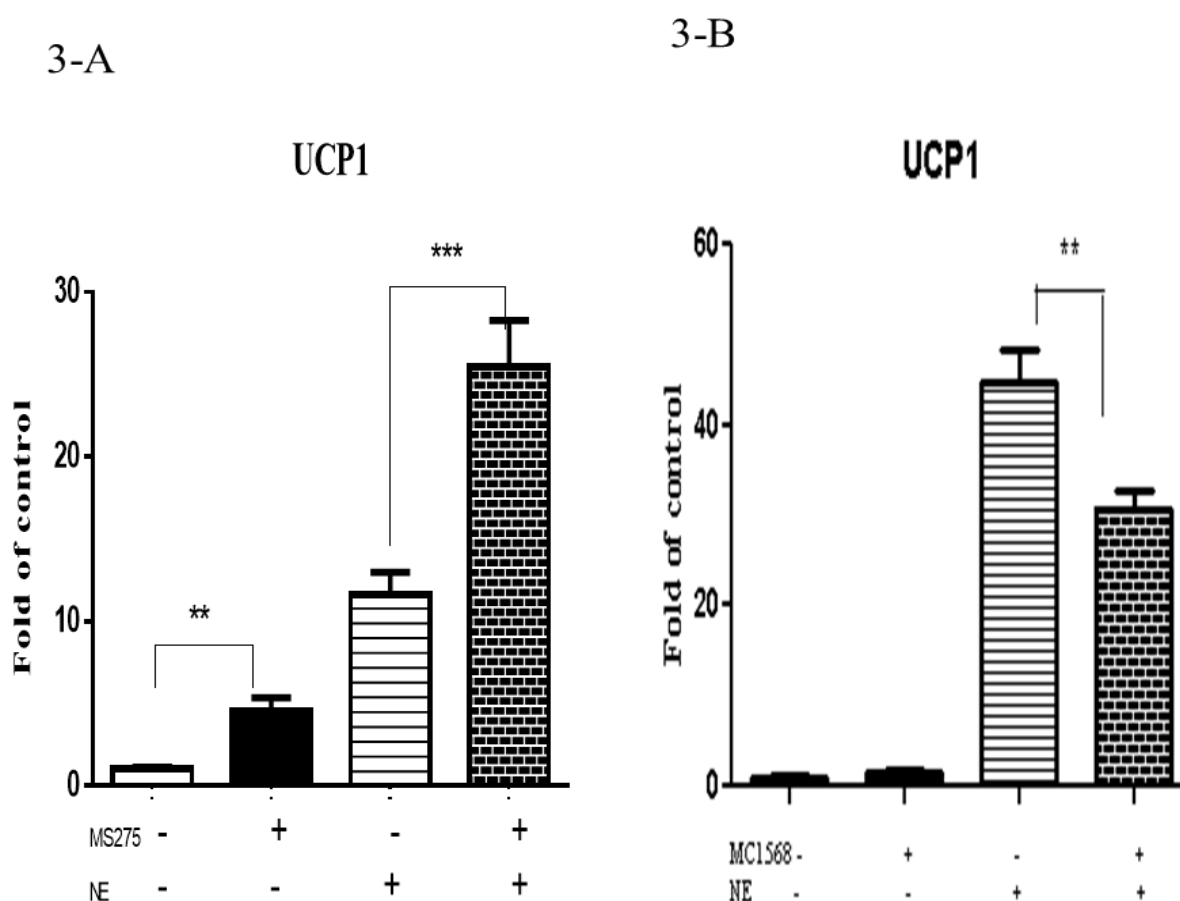


Fig 3 UCP-1 expression in H1B1B cell lines after treatment with different classes of HDAC inhibitor using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (norepinephrine) for 4 hours to observe changes in thermogenic gene, UCP1. 3A): Up-regulated by Class-I inhibitor- MS 275 and 3B) Down-regulated by Class-II inhibitor MC 1568. Values in the experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=4-6). \*\*P < 0.01, \*\*\*P < 0.001

**Figure 3: UCP1 expression in H1B1B cells.**

### **4.3 Effects of Class-I HDAC inhibitor in brown adipocytes**

Similar to its effect on UCP1 expression, the treatment of Class-I HDAC inhibitor MS-275 in H1B1B cells also promoted the gene expression of transcriptional activators of UCP1 such as: PGC1 $\alpha$ , PGC1 $\beta$  and PPAR $\gamma$ . It also increased the expression of ACOX-1 and CIDEA, which are associated with oxidation of fatty acids and lipolysis respectively (Fig: 4). These effects are similar to that of TSA in HIB1B cells. These data indicate that these HDAC inhibitors regulate the gene expression pattern differently.

### **4.4 Role of HDAC inhibitors in BAT1 cell lines**

To confirm the effect of HDAC inhibition on brown adipocytes, a different brown fat cell line, BAT-1 were used. The studies were repeated by pre-treating the cell lines with HDAC inhibitors for 30 minutes and followed by isoproterenol (adrenergic stimulation) for 3 hours. As seen in H1B1B cell lines, BAT1 cells exhibited a similar gene expression profile after treatment with HDAC inhibitors. In addition to UCP1 down-regulation, TSA also decreased the gene expression of PPAR $\gamma$ , PRDM16 and classical brown fat markers like deiodenase-2 (DIO2), elongation of very long chain fatty acids (ELVOL3), potassium channel, subfamily K, member 3 (KCNK3) and otopenin 1 (OTOP1) (Fig: 5A and 5B). In contrast, TSA in BAT1 cells significantly up-regulated PGC1 $\alpha$ , PGC1 $\beta$  and ACOX-1, similar to TSA's effect on these gene expressions in HIB1B cells.

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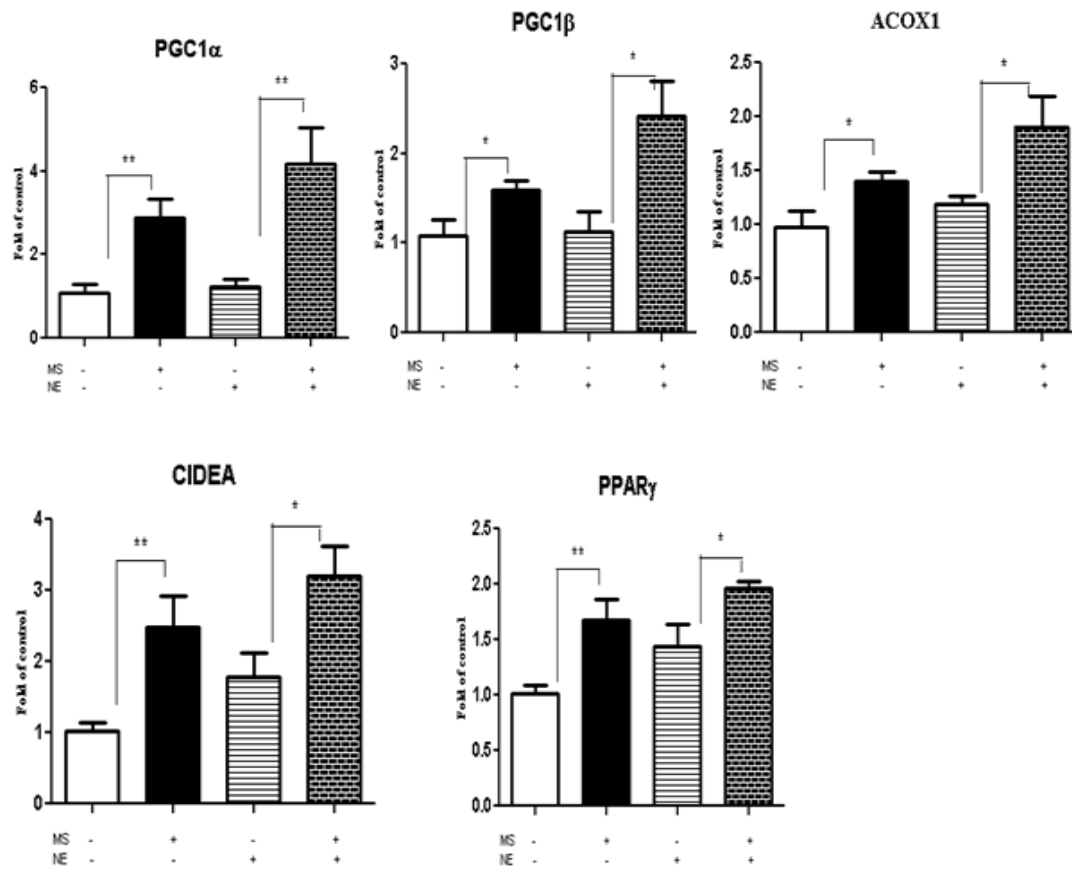


Fig 4: Effect of MS-275, a Class-I HDAC inhibitor on H1B1B cell lines using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (norepinephrine) for 4 hours to observe changes in thermogenic and regulatory genes. 4-A) Up-regulated genes Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=3-6). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

Figure 4: Effect of MS-275 in H1B1B cells.

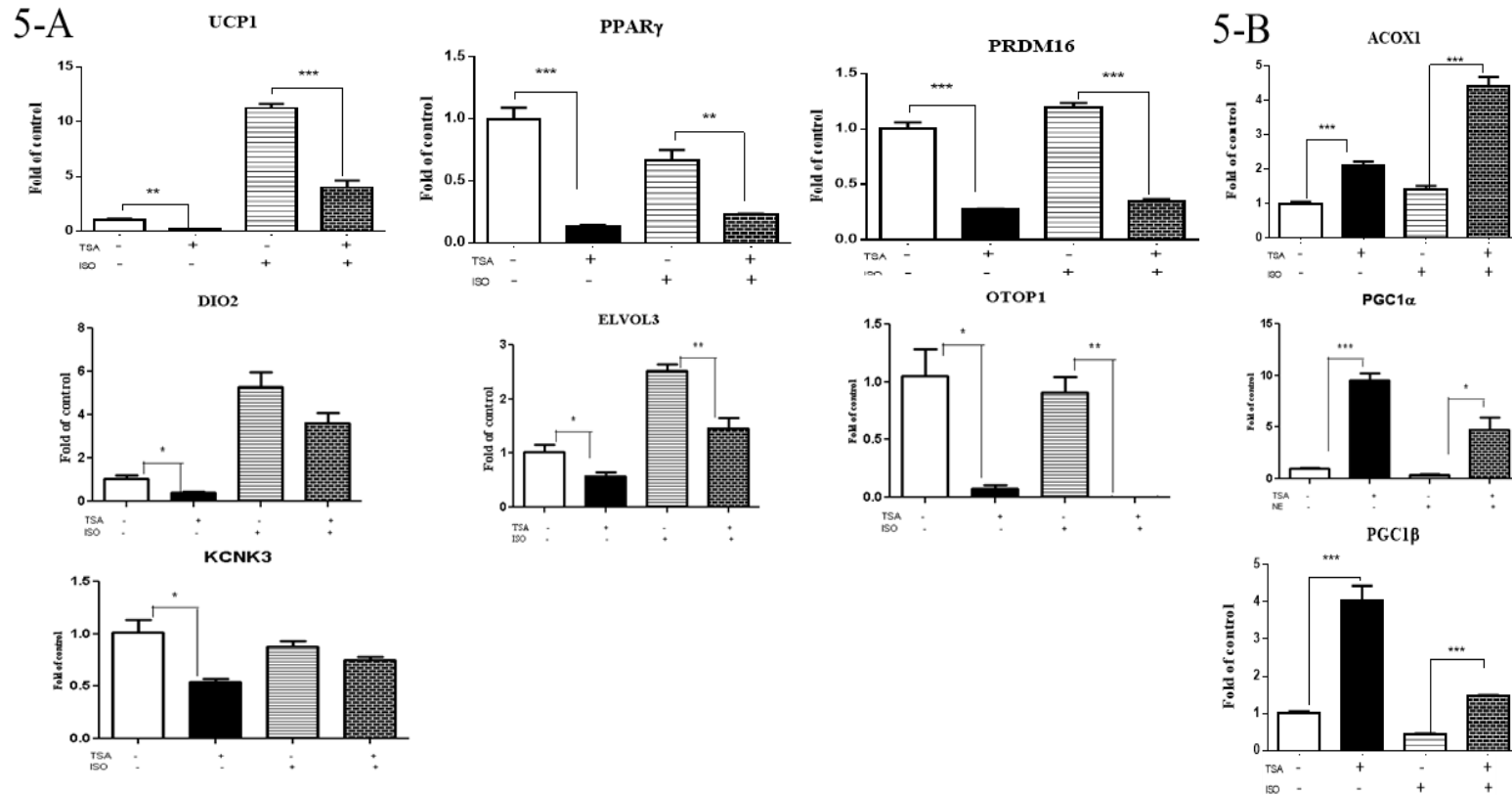


Fig 5: Effect of TSA, a pan HDAC inhibitor on BAT1 cell lines using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (isoprotorenol) for 3 hours to observe changes in thermogenic and regulatory genes. 5-A) Down-regulated genes 5-B) Up-regulated genes. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=3). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

**Figure 5: Effect of TSA in BAT-1 cells.**

Similar to H1B1B cell lines, BAT-1 cells also showed a significant decrease of UCP1 expression after Class-II HDAC inhibition and increase of UCP1 after Class-I HDAC inhibition (Fig: 6A and 6B).

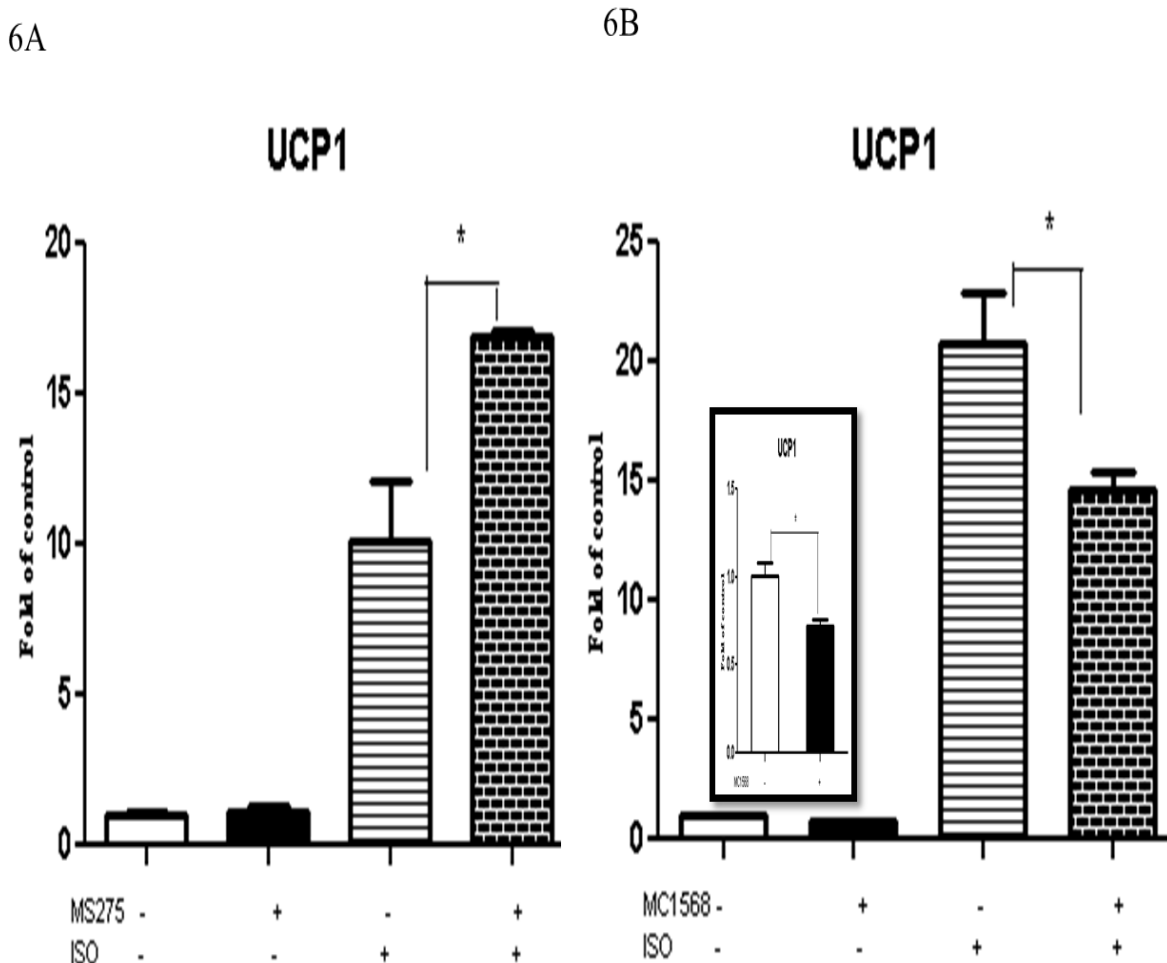


Fig 6 UCP-1 expression in BAT1 cell lines after treatment with different classes of HDAC inhibitor using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (isoprotorenol) for 3 hours to observe changes in thermogenic gene, UCP1. 6A: Up-regulated by Class-I inhibitor- MS 275 and 6B: Down-regulated by Class-II inhibitor MC 1568. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=3). \*P < 0.05.

**Figure 6: UCP1 expression in BAT1 cells.**



#### 4.5 Increase in brown fat specific genes after Class-I HDAC inhibition

Analogous to H1B1B cell lines, BAT-1 cells treated with Class-I HDAC inhibitor, MS-275 showed a similar gene expression profile pattern. In BAT-1 cells, MS-275 increased the gene expression of PGC1 $\alpha$ , COX-1, ACOX-1 and brown fat markers such as: ELVOL3 and DIO2 (Fig: 7). This suggests that inhibition of Class-I HDACs plays a crucial role in up-regulation of brown fat gene expression whereas inhibition of Class-II HDACs significantly suppresses UCP1 expression in brown adipocytes.

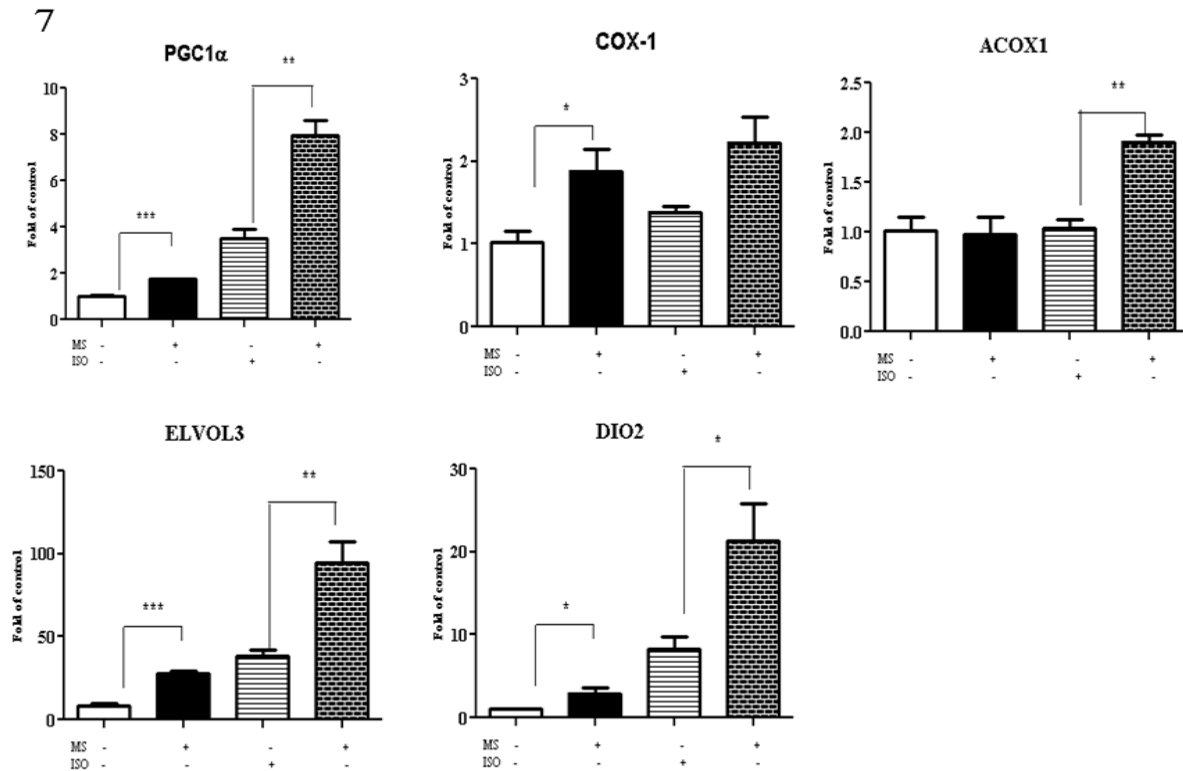


Fig 7: Effect of MS-275, a Class-I HDAC inhibitor on BAT1 cell lines using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (isoproterenol) for 4 hours to observe changes in thermogenic and regulatory genes. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=3). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

**Figure 7: Effect of MS-275 in BAT1 cells.**

To further investigate the effect of Class-I HDAC inhibitor in promotion of brown fat morphology, we treated white adipocytes at different time periods starting from 30 minutes to 4 days. 3T3-L1 adipocytes, a classical white adipocyte cell line showed an increase in UCP1 expression after treatment with MS-275 (Fig: 8). This clearly demonstrates the up-regulation of UCP1 expression by Class-I HDAC inhibition.

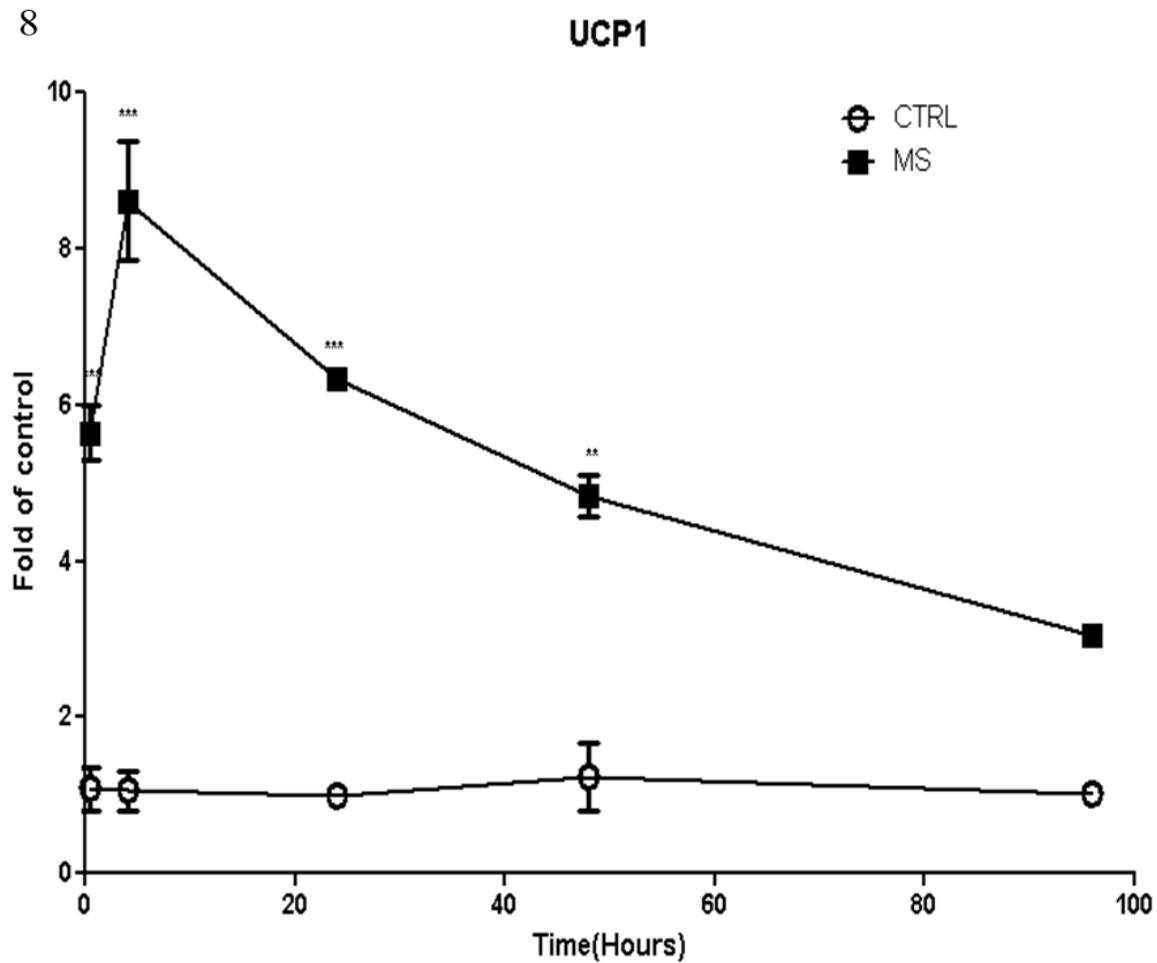


Fig 8: Effect of MS-275, a Class-I HDAC inhibitor on UCP1 gene expression in 3T3 L1 cell lines using RT-PCR analysis. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=3). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

**Figure 8: Effect of MS-275 on UCP1 expression.**

#### **4.6 Class-II HDAC inhibitors target Rb1 to repress UCP1 expression:**

H1B1B and BAT-1 cell lines treated with TSA and MC-1568 showed a significant decrease of UCP1 expression and a robust increase of Rb1 expression. This suggests that TSA behaved more like a Class-II HDAC inhibitor in down-regulation of UCP1 expression and up-regulation of Rb1 expression. In contrast, the Class-I HDAC inhibitor (MS-275) had a very minor or no significant effect on Rb1 expression (Fig: 9A and 9B). Since Rb1 is a negative regulator of UCP1 expression, we hypothesize that this robust increase of Rb1 expression by TSA and MC-1568 likely contributes to the inhibitory effects of TSA and MC-1568 on UCP1 expression.

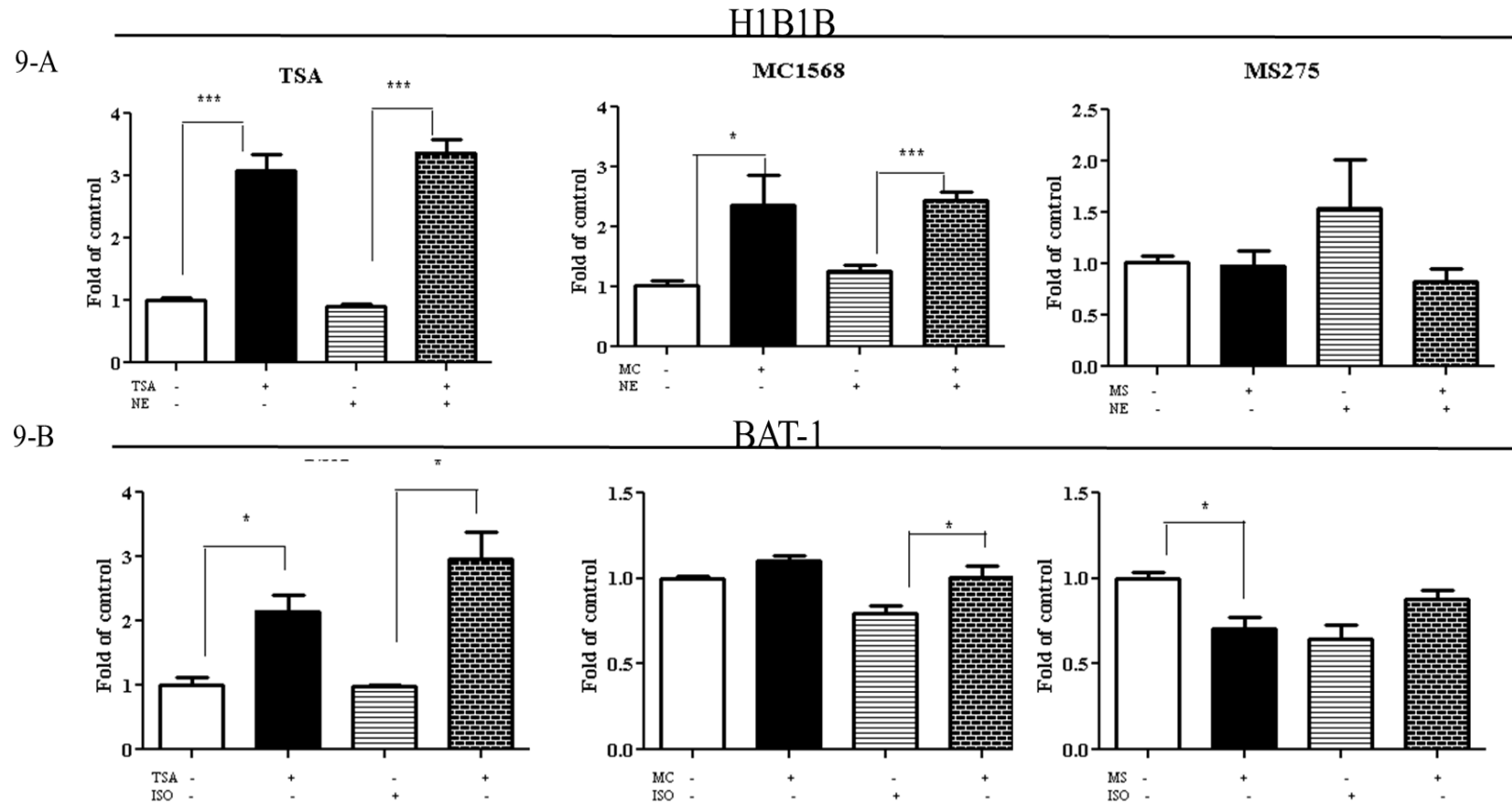


Fig 9 RB-1 expression in brown fat cell lines after treatment with different classes of HDAC inhibitor cell lines using RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (norepinephrine/isoproterenol) for 3-4 hours to observe changes in the regulatory gene, Rb1. 9A: H1B1B cell lines and 9B: BAT-1 cell lines. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n=3-6). \*P < 0.05, \*\*\*P < 0.001

**Figure 9: Rb1 expression after HDAC inhibitors treatment.**

#### **4.7 Rb1 mediated epigenetic regulation of UCP1 expression by TSA and MC-1568.**

TSA inhibits HDACs, which in turn increases the acetylation levels on histone lysine sites<sup>47</sup>. We assessed the changes in histone acetylation status of Rb1 promoter region. Using ChIP analysis, we studied the histone acetylation pattern of H3K27 residues on Rb1 promoter in BAT-1 cells after treatment with TSA and MC-1568. Treatment with TSA and MC-1568 enriched acetylated H3K27 at the promoter region of Rb1, which may in turn, be responsible for the increased Rb1 expression observed after TSA and MC-1568 treatment. This increased expression of Rb1 may then inhibit UCP1 expression<sup>17</sup>. This demonstrates how the treatment with Class-II HDAC inhibitor suppressed UCP1 expression by recruiting more acetylated histone groups on repressor genes, such as Rb1 promoter regions and thus promoting its gene expression (Fig: 10).

In line with ChIP results, to confirm that increase in Rb1 expression is responsible for suppression of UCP1 expression, we performed siRNA knockdown of Rb1 in brown adipocytes. After siRNA knockdown, Rb1 expression was decreased by 70% (Fig: 11A). After knockdown of Rb1, UCP1 expression was significantly increased at both basal level and isoproterenol treatment level (Fig: 11B). In addition, Rb1 knock down completely reversed the effect of TSA on inhibiting UCP1 expression when treated with isoproterenol. Rb1 knockdown also reversed the effect of MC-1568 on inhibiting UCP1 expression both at the basal level and isoproterenol stimulated condition.

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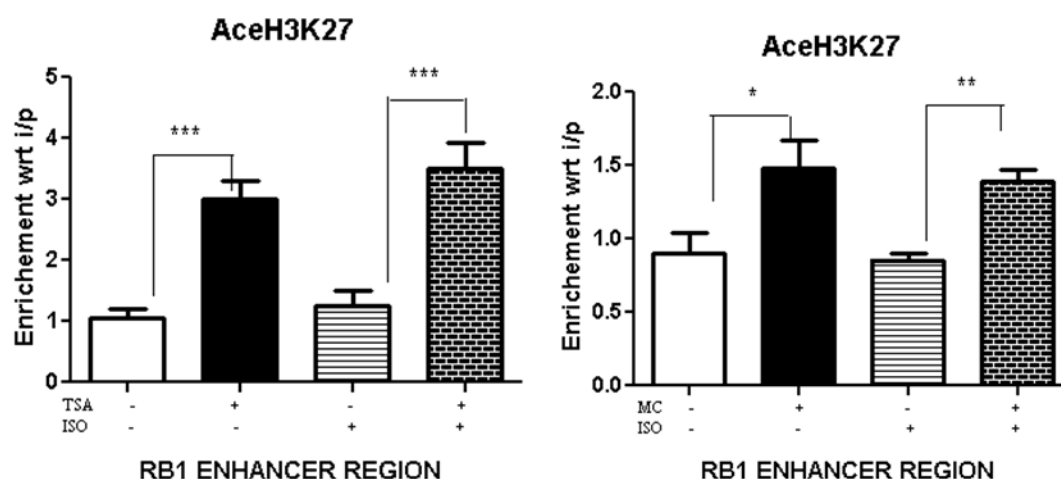


Fig 10: Class-II HDAC suppress UCP1 expression by up-regulation of histone acetylation at the Rb1 site. Enrichment of the specific histone residue, aceH3K27 at Rb1 promoter region after treatment with TSA and MC1568. ChIP and RT-PCR analysis was performed to study the histone acetylation status. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (isoproterenol) for 3 hours to observe changes in enhancer regions of regulatory gene, Rb1. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n>4). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001

**Figure 10: Effect of HDACs on Rb1 promoter region.**

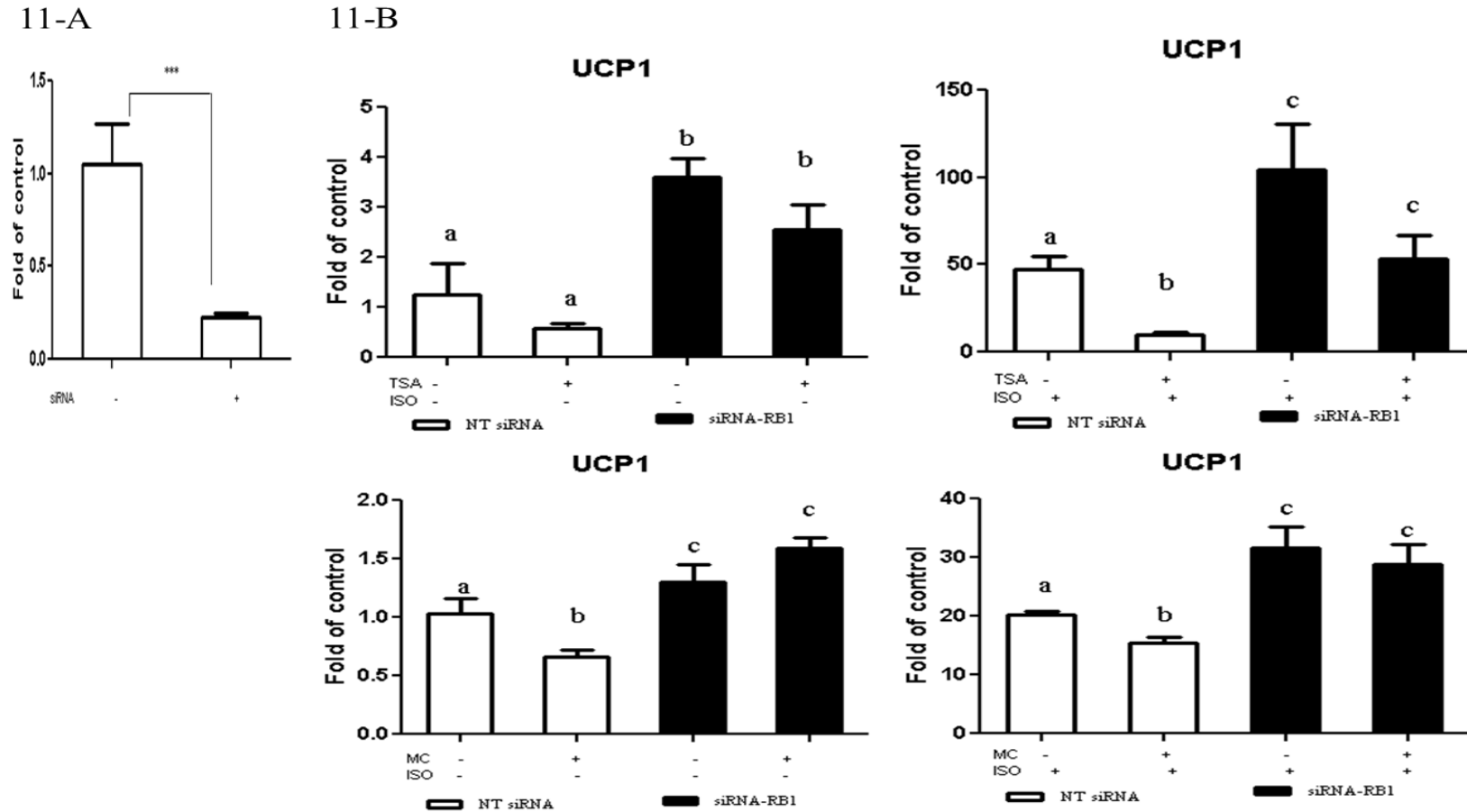


Fig. 11: Pan HDAC inhibitor, TSA and Class-II HDAC inhibitor, MC1568 exerts its effect on UCP1 through Rb1. 11-A: Knock down efficiency of Rb1 siRNA, data are presented as means  $\pm$  SE (n=4) \*\*\*P < 0.001. 11 B: A significant up-regulation of UCP1 expression in BAT-1 cells after Rb1 knock down when treated with TSA and MC-1568. Values in each independent experiment were normalized to control group (defined as one fold) and groups labeled as superscripts are statistically different from each other, p<0.05

**Figure 11: Effect of Rb1 knockdown in UCP1 expression.**

#### 4.8 Mechanism of Class-I HDAC inhibition

There are evidence from literature that, PGC1 $\alpha$  is important in up-regulation of UCP1 expression<sup>2, 12, and 25</sup>. To explore the mechanism of Class-I HDAC inhibition in the up-regulation of UCP1 expression, we treated cells with MS-275 and performed ChIP assay to study the acetylation status of PGC1 $\alpha$  transcriptional regions: Mef2 (Myocyte enhancer factor) and CREB (cAMP response element binding region) binding sites. The CREB- and Mef2-binding sites are two important cis-elements on PGC1 $\alpha$  promoter that transactivate its expression<sup>25, 26, and 27</sup>. Our results indicate a significant recruitment of acetyl groups to both CREB and Mef2 sites, with CREB-binding site showing the most significant increase in acetylation status over Mef2 binding region (Fig: 12A). In addition, MS-275 also promoted acetylation of H3 residues on UCP1 start and enhancer regions with UCP1-start region showing the most significant effect over the UCP1-enhancer region<sup>40</sup> (Fig: 12B).



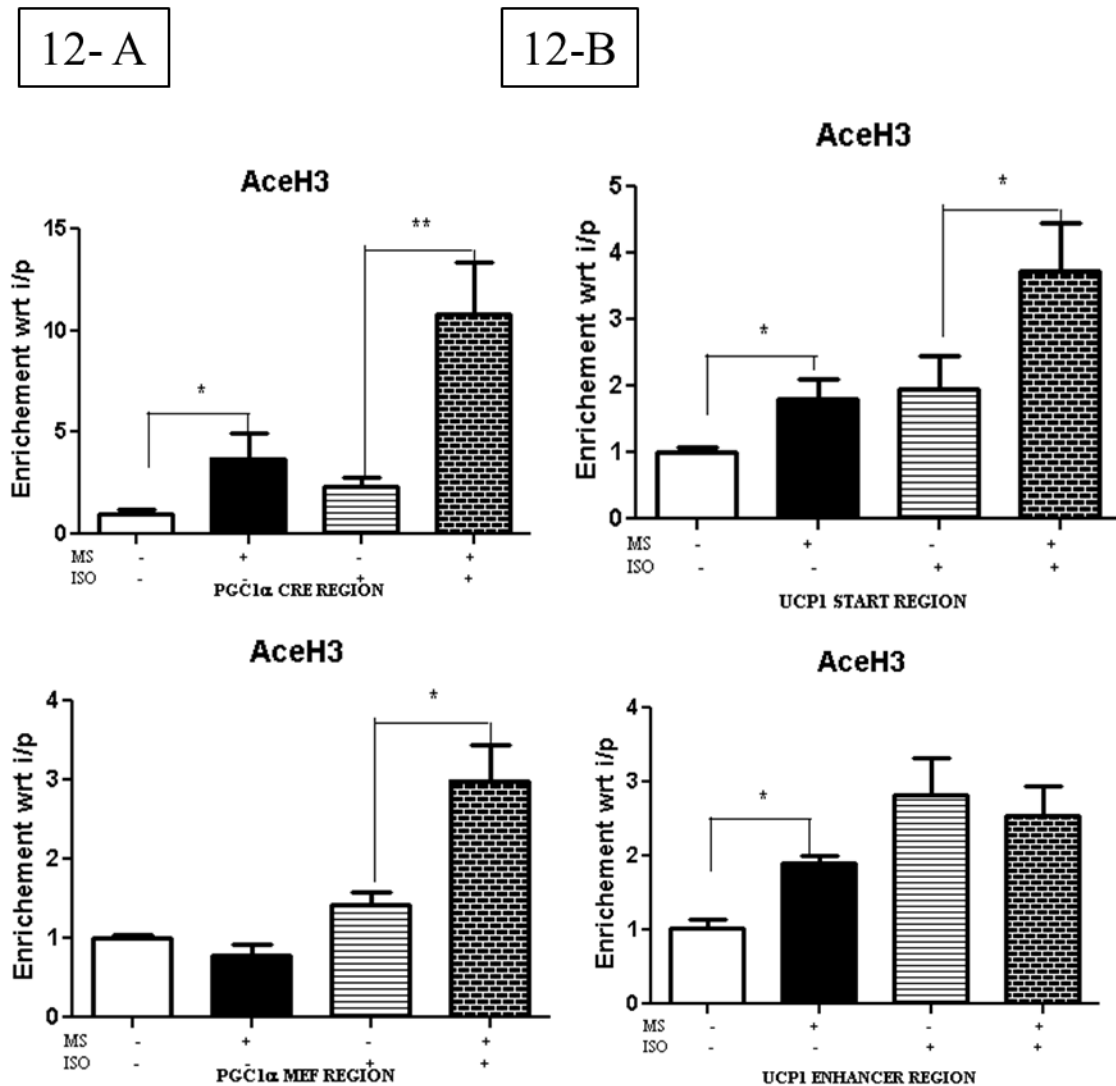


Fig 12: Class-I HDAC alleviates UCP1 expression through PGC1 $\alpha$ . 12A: Effect of MS275 at PGC1 $\alpha$  MEF and cre region and 12B: Effect of MS275 at UCP1 enhancer and start using ChIP and RT-PCR analysis. Cells were pretreated with HDAC inhibitor for 30 minutes followed by adrenergic stimulation (isoproterenol) for 3 hours to observe changes in enhancer regions of thermogenic gene, UCP1. Values in each independent experiment were normalized to control group (defined as one fold) and data are presented as means  $\pm$  SE (n>4). \*P < 0.05, \*\*P < 0.01.

**Figure 12: ChIP analysis after MS-275 treatment.**

The role of PGC1 $\alpha$  in mediating the effect of MS-275 in UCP1 expression is verified by siRNA knockdown of PGC1 $\alpha$ . The knockdown efficiency was more than 70% and it significantly reduced UCP1 expression both at basal level and isoproterenol treatment level. It also significantly prevented the increase in UCP1 expression after treating the cells with the compound, MS-275. This suggests that (i) PGC1 $\alpha$  is very important for regulating UCP1 expression (ii) PGC1 $\alpha$  is mediating MS-275 effect on up-regulating UCP1 expression (Fig: 13).

13-A

13-B

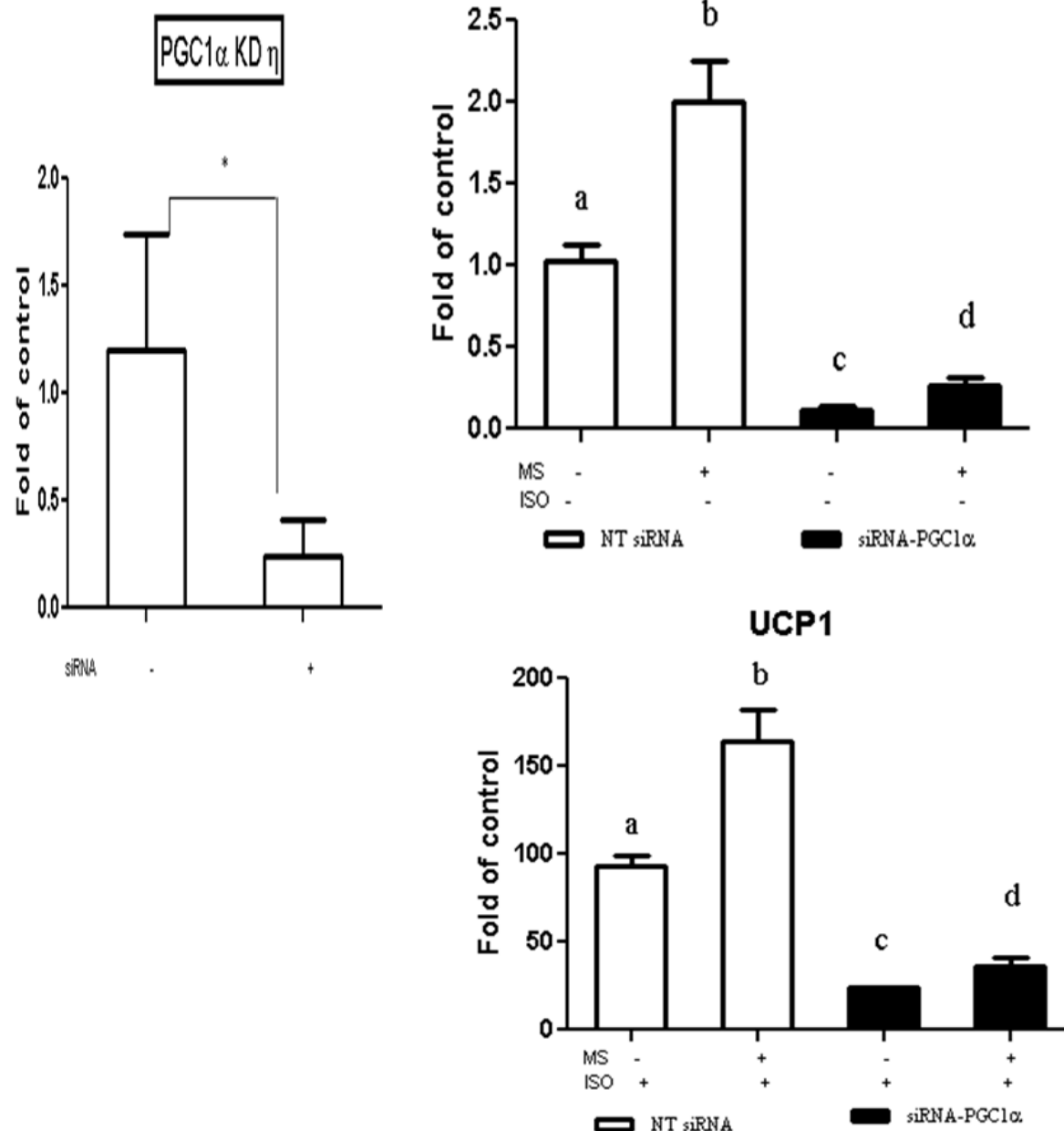


Fig. 13: Class-I HDAC inhibitor MS-275 exerts its effect through PGC1 $\alpha$ . 13-A: Knock down efficiency. 13-B: A significant down regulation of UCP1 after PGC1 $\alpha$  knock down. Values in each independent experiment were normalized to control group (defined as one fold) and groups labeled as superscripts are statistically different from each other,  $p < 0.05$

**Figure 13: Role of PGC1 $\alpha$  knockdown in UCP1 expression.**

## 5 DISCUSSION

It is long known that sympathetic activation is important for UCP1 expression. For example: the activation of  $\beta_3$  adrenergic receptor can up-regulate the protein kinase A (PKA) signaling pathway, which promotes UCP1 gene expression<sup>43, 44</sup>. In addition, there are important transcriptional regulators of UCP1 such as: PGC1 $\alpha$ , PGC1 $\beta$ , and PRDM16, which are known to regulate UCP1 expression. Thus, over expression of PGC1 $\alpha$  or PRDM16 can also promote UCP1 expression<sup>45, 46</sup>. There are evidences that support modification in histone acetylation pattern and other epigenetic modifications like DNA methylation can also up-regulate UCP1 expression<sup>6,7,10</sup>. However, in spite of the large amount of informations available towards the regulation of brown fat function by these signaling pathways and transcriptional regulators, specific information on epigenetic regulation of brown fat metabolism remains unclear. A recent study on Class-I HDACs revealed its role on oxidative metabolism in skeletal muscle<sup>10</sup>. Other previous studies on butyrate, which blocks HDAC function have also reported a positive role of HDAC inhibition in energy expenditure<sup>28, 29 and 30</sup>. These well-established studies provide evidence that, HDACs play an important role in energy metabolism.

However the detailed mechanisms of how histone acetylation regulate UCP1 expression is unclear. Through our study, we have addressed this question by providing a direct evidence for the mechanism of UCP1 regulation after HDAC inhibition. Although literature provides us with some insight about the role of Class-I HDACs in adipocytes and myocytes<sup>10</sup>, the exact mechanism is still unknown. In addition, the effect of Class-II HDAC in the regulation of UCP1 expression is unclear.

In our study, we used a pan HDAC inhibitor-TSA, Class-I HDAC inhibitor-MS-275 and Class-II HDAC inhibitor- MC-1568 on various adipocyte cell lines to understand the molecular basis and epigenetic mechanisms of the regulation of UCP1 expression by different HDACs. Our efforts were focused on identifying both the activators and repressors of UCP1 induced by epigenetic modulation by HDACs.

Our results show that TSA, a pan inhibitor of HDACs, and MC-1568, a class II HDAC inhibitor, significantly down-regulated UCP1 expression, whereas MS-275, a class I HDAC inhibitor, significantly up-regulated UCP1 expression. In addition, while TSA behaves more like a class II HDAC inhibitor in inhibiting UCP1 expression, TSA up-regulates PGC1 $\alpha$  expression, similar to that of the class I HDAC inhibitor, MS-275. These data suggest that different HDACs may exert different effects regulating brown fat gene expression.

Treatment with MS-275 inhibits the Class-I HDACs and our data suggests that it increases UCP1 expression in brown adipocyte cell lines (Fig: 3 and 6). This increase in UCP1 expression is correlated with increase in its activator: PGC1 $\alpha$  (Fig: 4 and 7). To further evaluate the role of MS-275 in up-regulation of PGC1 $\alpha$ , we performed ChIP on PGC1 $\alpha$ . Previously identified transcriptional enhancer regions of PGC1 $\alpha$ : Mef and CRE sites were targeted for our ChIP experiments<sup>10, 25, 26 and 27</sup>. Our results on inhibition of Class-I HDACs by MS-275 showed increased acetylation on both the sites and a more significant effect was seen at the CRE site (Fig: 12A). This enrichment of histone acetylation at PGC1 $\alpha$  transcription region, after treatment with MS-275 is consistent with the previous work done by Galmozzi et al<sup>10</sup>.

In our study, we have also shown that MS-275 can directly promote acetylation at the UCP1 transcriptional regulatory and start region. From previous established data, we know that UCP1 transcriptional regulatory region is present around 2.8 kb from its start site<sup>38 and 39</sup>. This region includes binding motifs like: CRE, PPRE (peroxisomal proliferator activator receptor binding motif), BRE (brown adipocyte regulatory element) and also some negative regulatory elements, such as Rip-140 binding site. Our results indicate that MS-275 can enrich histone acetylation at this UCP1 enhancer region, as well as at the UCP1 transcriptional start region (Fig: 12B). We believe, MS-275 can directly act on UCP1 and as well act on UCP1 positive regulator, PGC1 $\alpha$  to up-regulate its expression. Apart from this, MS-275 also increased a whole panel of brown fat specific genes and thus promoting brown adipocyte function (Fig: 4 and 7). Not only in brown adipocyte cell line, but also in white adipocyte cell line, MS-275 significantly up-regulated UCP1 expression (Fig: 8), indicating that MS-275, by inhibiting Class-I HDACs, may also be important in the browning of white adipocytes. This compound, MS-275 in animal model showed a physiological significance in ameliorating obesity and promoted up-regulation of PGC1 $\alpha$  in skeletal muscles in other studies<sup>10</sup>. Based on all of these evidences and our results, we interpret that inhibition of Class-I HDACs play a vital role in enhancing brown adipocyte function, and in browning of white adipocytes and thus induction of beige cell phenotype in white adipocytes.

To confirm that treatment with MS-275, exerted its effect on UCP1 by directly targeting PGC1 $\alpha$ , we performed knockdown of PGC1 $\alpha$  in brown adipocytes.. After knockdown, we observed that effect of MS-275 on UCP1 up-regulation was significantly reversed. Thus, the inhibition of Class-I HDACs increased the acetylation status of PGC1 $\alpha$  and as well as increased

the acetylation status of both UCP1 start and enhancer region. Taken together, these epigenetic changes significantly up-regulate UCP1 expression.

On the other hand, both TSA and Class-II HDAC inhibitor, MC-1568 significantly down-regulated UCP1 expression. We hypothesize, the inhibition of HDACs by TSA and MC-1568 activate certain repressor genes that are responsible for the suppression of UCP1 expression. From literature, we know that Rb1 and RIP140 are well documented negative regulators of UCP1<sup>11, 12, 17 and 18</sup>. Our results indicate that all the HDAC inhibitors used in our study: TSA, MS-275 and MC-1568 up-regulated RIP140. Whereas only MC-1568 and TSA up-regulated Rb1, while MS-275 had no effect on Rb1 expression (Fig: 9). Based on this result, we expect Rb1 to be a likely target for Class-II HDAC inhibitors to down-regulate UCP1 expression. Therefore, we focused the rest of our experiments on Rb1.

Rb1, which is primarily known as a tumor suppressor gene, is also known to regulate apoptosis, cell proliferation and differentiation<sup>31-33</sup>. In rodents, Rb1 is known as a negative regulator of brown adipocyte differentiation<sup>34</sup>. The inactivation of Rb1 is thought to induce brown fat like features in white adipocytes. In literature, however there is no clear evidence how Rb1 gene expression can be regulated by epigenetic mechanisms, which in turn controls UCP1 expression.

Our ChIP results clearly demonstrated that inhibiting Class-II HDACs recruited more acetyl groups on Rb1 promoter region which may in turn up-regulate its expression (Fig: 10). This indicates that inhibition of Class-II HDACs promoted Rb1 gene expression and Rb1 being a

negative regulator of UCP1, can suppresses UCP1 expression by negatively regulating PKA-CREB pathway<sup>17</sup>.

The increase in Rb1 expression can indirectly inhibit up-regulation of UCP1 expression by inhibiting the PKA signaling cascade. PKA signaling cascade can be activated by sympathetic stimulation. When adrenergic agonists, such as norepinephrine and isoproterenol bind to their G-Protein Coupled Receptor ( $G_s$ ), the  $G_\alpha$  subunit is released to activate adenylate cyclase (AC). AC can catalyze the generation of cAMP, which binds to PKA to release the regulatory subunit from the catalytic site. The free, activated PKA catalytic unit can now diffuse inside the nucleus to phosphorylate and activate cAMP Response Element Binding Protein (CREB). The activated CREB can act on its downstream target or the promoter regions of various genes to up-regulate their expression. For example, CREB binds directly to UCP1 promoter to activate its gene expression<sup>38 and 39</sup>.

From literature, we know that transcription of Protein Kinase A can be differentially regulated by either up-regulating the synthesis of regulatory subunits or its catalytic subunit<sup>35</sup>. Cell utilizes this function to regulate the PKA signaling cascade. Rb1 increases FOXC2 (Forkhead box protein C2), which is a transcription factor belonging to FOX (Forkhead box) family. This FOXC2 can then increase PKA-R1 $\alpha$  (Protein Kinase A- Regulatory Subunit-I $\alpha$ ) expression, which is a negative regulator of PKA signaling cascade. Thus up-regulation of Rb1 expression via enriched histone acetylation on its promoter can in turn increase the PKA-R1 $\alpha$  subunit. This can suppress UCP1 expression by inhibiting the PKA signaling pathway (Fig 14).



This is indeed consistent with literature that up-regulation of Rb1 increases PKA-R1 $\alpha$ , which inhibits the upstream signaling of  $\beta$ -agonist to decrease PKA activity<sup>17</sup>.

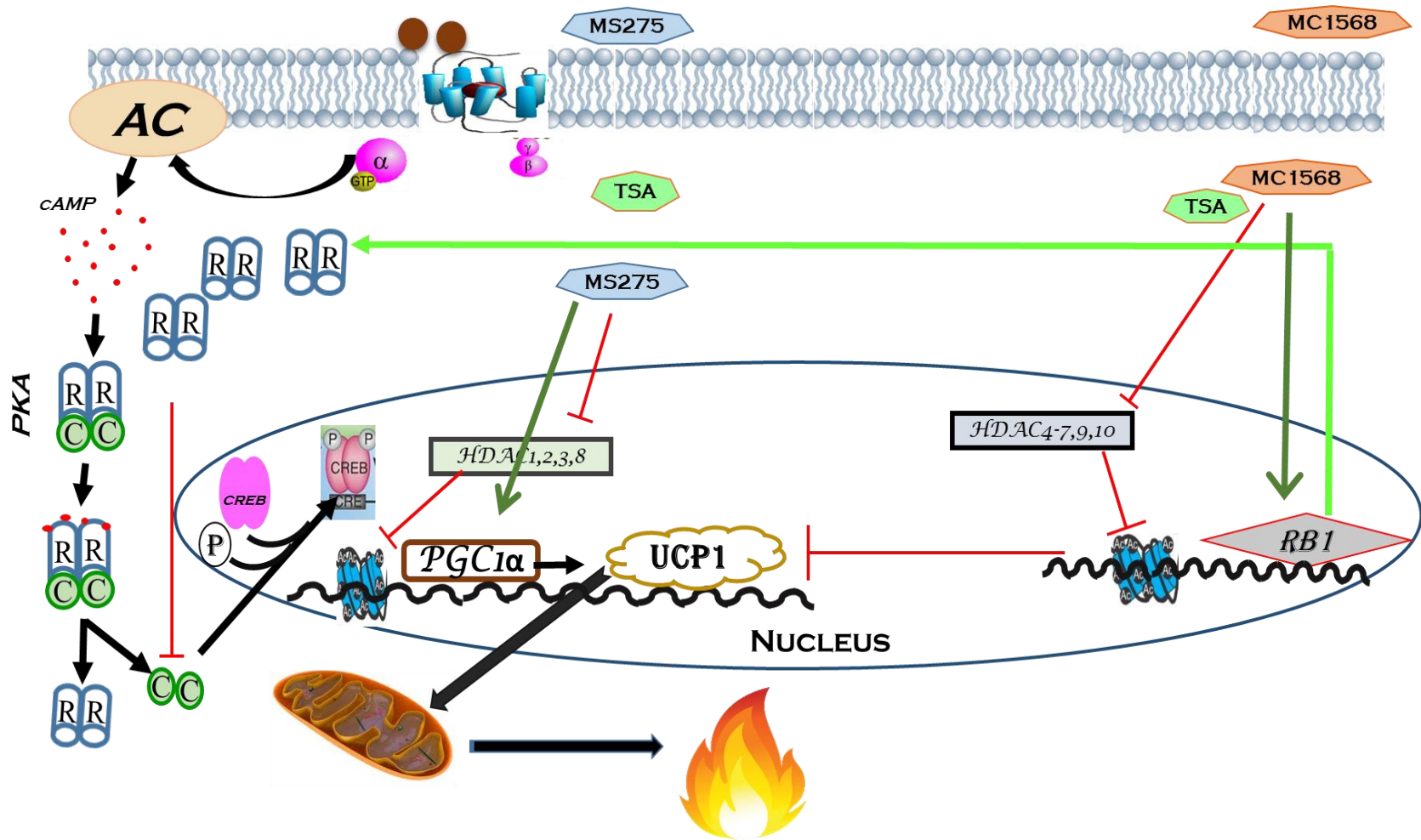


Figure 14: A cartoon depicting the mechanism of different mode of action by different HDAC inhibitors.

The following evidences support our hypothesis that Rb1 mediates the effect of Class-II HDAC inhibitors in suppression of UCP1 expression: i) significant increase in Rb1 expression after TSA and MC-1568 treatment, which is consistent with significant decrease in UCP1 expression ii) enrichment of H3K27 acetylation at Rb1 promoter region to up-regulate its expression iii) the ability of Rb1 knockdown to reverse the effect of TSA and MC-1568 in suppression of UCP1. In summary, all these data clearly demonstrates that Rb1 mediates suppression of UCP1 expression by TSA and MC-1568 in brown adipocytes. To our knowledge, this is the first study which links the epigenetic modulation of Rb1 in regulation of UCP1 expression.

In conclusion, we believe that there exists two different regulatory mechanism of UCP1 expression. Class-I HDAC inhibition can up-regulate UCP1 expression through PGC1 $\alpha$  and Class-II HDAC inhibition can down-regulate UCP1 expression through Rb1. To the best of our knowledge this is the first study which links the epigenetic regulation of UCP1 via HDAC inhibition.

In spite of understanding the mechanism of Class-I and Class-II HDACs on UCP1 expression, the role of specific HDACs in regulation of UCP1 is yet to be studied. This will further help us understand how epigenetics control brown fat thermogenesis and what role it plays in energy balance. By targeting the specific HDACs using synthetic or natural drugs, it might be possible to develop a promising therapy towards treatment of obesity and its associated disorders.

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